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Antimicrobial *in vitro* activities of condensed tannin extracts on avian pathogenic *Escherichia coli*

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Significance and impact of the study

This study showed that condensed tannins (CTs), which were a group of secondary metabolites of many plants and rich in prodelphinidins (PD), had greater antibacterial activity against avian pathogenic *E. coli* (APEC) than CTs that were rich in procyanidins (PC). The mode of action of the CTs was to inhibit the swimming and swarming motility of APEC, and its ability to form biofilms. The significance of this finding is that the use of PD-rich CTs to control APEC should not encourage the development of antibiotic resistance by APEC because a different mechanism is used. If confirmed *in vivo*, this could provide the poultry industry with a valuable and novel means of controlling the antibiotic resistance.

Abstract

Condensed tannins (CTs), which extracted from yew leaves, tilia flower and black locust leaves, were examined for their antimicrobial *in vitro* activity against avian pathogenic *Escherichia coli* (APEC). Past research demonstrated that CTs which contain procyanidins and prodelphinidins that could inhibit the growth of a wide range of bacteria. However, there is no information on how these affect pathogenic bacteria from chickens such as APEC.

The high concentration of extracts, 10, 5, 2.5 mg/ml, affected the growth curves of APEC, which gave different inhibition values for the three CT extracts. Further, these

CTs had significant effects (P≤0.05) on APEC biofilm and motility depending on each CT concentration and composition. However, at low concentration (0.6 mg/ml), the tilia flowers, a high molar percentage of procyanidins, enhanced bacterial cell attachment and improved the swimming motility of APEC. In contrast, yew, an equal molar percentage of procyanidins/prodelphinidins, and black locust, a high molar percentage of prodelphinidins, interrupted and blocked swarming and swimming motility. The data suggested that the antimicrobial activity of the CT extracts was elicited by a positive relationship between anti-biofilm formation and anti-motility capacities.

Keywords: Condensed tannins, avian pathogenic *Escherichia coli,* antimicrobials, biofilm, motility.

Introduction

The emergence of antibiotic resistance led to the banning of antimicrobial agents in feeds as growth promoters in Europe (Dibner and Richards 2005; Koluman and Dikici 2013). Antibiotic addition to feeds has also been found to affect intestinal microflora (Niewold 2007), which have been increased the demands for effective substances to reduce pathogenic bacteria and improve animal health (Kroismayr *et al.* 2008). Last decade, numerous reports demonstrated the development of antibiotic resistance that started to impact negatively on our ability to treat some human pathogens (Karikari *et al.* 2017). Thus, medicinal plants and herbs are being investigated as a potential solution to promote animal performance without fostering antibiotic resistance (Baurhoo *et al.* 2007). Many natural plant products possess antimicrobial activities (Windisch *et al.* 2008; Liu *et al.* 2011) and have been incorporated into animal feeds as supplements instead of synthetic drugs. One example of such products are tannins, which are produced as part of the secondary metabolism of several higher plants (Frutos *et al.* 2004).

Escherichia coli is a diverse species that causes diarrheal disorders and a variety of gastrointestinal infections (Kaper *et al.* 2004). Some of these strains have demonstrated an ability to penetrate the mucus layer and efficiently colonise the mucosa of the large intestine (Torres *et al.* 2005). Therefore, *E. coli* has been one of the most important Gram-negative bacteria for *in vitro* experiments to form the biofilm on host surfaces (O'Toole *et al.* 2000; Van Houdt and Michiels 2005).

One particularly problematic *E. coli* species is avian pathogenic *Escherichia coli* (APEC), which can survive in different environments and induce infections in chickens, turkeys and other birds. These bacteria can cause aerosacculitis, polyserositis, septicaemia and other extraintestinal disorders (Giovanardi *et al.* 2013). *E. coli* have flagella that contribute to motility dependent upon the environment and can be an essential part of the induction of adhesion of microbes on a host surface enabling biofilm formation (Verstraeten *et al.* 2008). Motility can play a critical role in primary interference with a surface and can help these bacteria to promote biofilm development (Kearns 2010). There is evidence that bacteria can use various strategies to initiate biofilm formation, and it is not surprising that bacteria commonly utilise their cell structures such as flagella in motile stages (Pratt and Kolter 1998). Moreover, one of these virulence factors is polysaccharide capsule, which enable the bacteria to avoid the host immune-system (Alkandhari 2018). Therefore, more information on the effect of plant tannins on virulence factors should be considered. Furthermore, due to the evolution of antibiotic-resistant strains, this study investigated the antimicrobial activity of naturally occurring plant tannins as these could be of interest in the form of feed additives for the management of chicken pathogens. In particular, this study investigated the ability of CTs to interfere with APEC microbial activities such as growth, biofilm formation and motile activity in *in vitro* experiments.

In conclusion, this study investigated the effect of CT concentrations and structural features on APEC growth, biofilm formation and motility. Significant antibacterial effects of CTs against APEC were observed, particularly if the CTs were rich in PDs. These findings may provide opportunities for use of PD-rich CTs in the management of bacterial diseases, such as colibacillosis in chickens. This will require further studies to optimise CT preparations and to evaluate them against a wide range of bacterial strains under farm conditions. In the present work, relatively high CT concentrations were used and showed antimicrobial activities against APEC by affecting the growth, biofilm formation and motility. However, low concentrations (0.6 mg/ml) of some CTs, particularly the procyanidins, had either a weak effect on antimicrobial activity or even enhanced bacterial growth.

Results and Discussion

Impact of CTs on bacterial growth

This study explored the effects of three types of CTs, which presented their compositions in (Table 1). The CTs from tilia flowers consisted of high procyanidins (i.e. approximately 960 mg/g PC), yew leaves had CTs with a mixture of procyanidins and prodelphinidins (i.e. approximately 520 mg/g PC and 480 mg/g PD), and black locust CTs were mostly prodelphinidins (760.9 mg/g PD).

These CTs were tested against APEC growth using a microtiter broth dilution method. Figure 1 shows the effect of different concentrations of CTs, including high PD of black locust, medium PC/PD of yew and high PC of tilia flowers on growth curves of APEC compared to the control. Irrespective of the source and composition of the CTs, similar patterns of inhibition were observed with the highest concentration, 10 mg/ml, causing complete inhibition. Interestingly, low concentration (0.6 mg/ml) of CTs extracted from tilia flowers appeared to slightly enhance the growth of APEC. Moreover, tilia flowers (high PC content) was statistically significant $P \leq 0.05$ at this concentration compared to control. This is intriguing and suggests that PC have less effect than PD compositions on bacteria, possibly because the number of hydroxyl groups is lower in the PC type than in the PD (Dakheel 2018). Generally, the growth curves demonstrated a dependency on CT concentrations, with the higher the CT concentration the lower the growth. Thus, the proportion of PDs within CTs was the most important parameter that influenced the biological activities of microorganism. However, it is also possible that the growth was similar but that the bacterial cell sizes were different; as this is the parameter that is measured (light refraction) by the spectrophotometer. This can be assessed by Electron Microscopy studies.

This study agreed to other studies that revealed the antimicrobial activity of several plants which are rich in tannins on a number of bacteria (Scalbert 1991; Doss *et al.* 2009). However, the present study reported the specific extracts of CT. The data generated in this paper showed inhibition but do not give any firm identification of the involved mechanism. However, a study reported by Holloway *et al.* (2015) concluded that catechin, and flavan-3-ols, which combined with inorganic compounds such as copper sulphate to generate hydrogen peroxide that would have an antimicrobial effect on pathogens.

APEC biofilm formation

The effect of CT concentrations and compositions on biofilm formation by APEC is illustrated in Figure (2). The high concentration of CT extracts (10 mg/ml) completely inhibited bacterial cell attachment of APEC ($P \leq 0.01$) because this concentration could be at the level of minimal bactericidal concentrations (MBCs), while other concentrations (5.0 - 1.25 mg/ml) displayed sub-MBC values of inhibition with significant differences ($P \leq 0.05$). This interesting finding could be explained that when the bacteria tried to survive, they adhered on the surfaces and formed the biofilm (Donlan and Costerton 2002). In contrast, the low concentration at 0.6 mg/ml of these CTs showed slightly enhancement of APEC but no significant differences ($P > 0.05$), except CT from tilia flowers (high PC content) that showed significantly ($P \leq 0.05$) different results at the low concentrations compared to the control. This result could indicate that plants with PD-rich CTs are more active against microbes than plants with high PC-rich of CTs.

Importantly, CT extract from black locust (high PD content) showed strong anti-biofilm activity, and no enhancement at the lowest concentrations compared to other CT extracts. Thus, low concentrations that are not inhibitory to APEC growth may contribute physically to increasing binding and biofilm formation. This is a novel finding that has not been reported before.

Based on the inhibitory results of the growth curve, above, the effect of CT on APEC was similar to biofilm finding. Although CTs inhibited biofilm formation which can protect bacterial cells from stressful factors such as antimicrobial agents (Bendaoud *et al.* 2011), the antimicrobial effect of these CT extracts combined to decrease of nutrients in the medium and this may stimulate biofilm formation as a survival strategy (Borges *et al.* 2012).

Inhibition of Motility

Figure 3 shows significant differences ($P \leq 0.05$) between the motility of APEC and different concentrations of CT extracts in a concentration dependent manner. The motility of APEC is less susceptible to PD than PC; this could probably be ascribed to some impact on their motile structures, e.g. flagella, as suggested previously (Pratt and Kolter 1998). A study reported that different tannin-containing plants can block the motility of bacteria (O'May and Tufenkji 2011). Therefore, our finding has been expanded to demonstrate that not only the concentration of CTs can influence motility

but also CT compositions can impact the motility of APEC as well. These results can be linked to the anti-biofilm effect of CT since bacterial motility plays an important role in adherence to surfaces and thus on the induction of biofilm formation and subsequent bacterial colonisation (Verstraeten *et al.* 2008).

This is the first study that demonstrates the effect of different concentrations and compositions of CT on blocking APEC motility in terms of swimming and swarming, which can cause the migrating bacteria to change direction. CTs showed different significant values ($P \leq 0.05$) on swimming and swarming activities. CTs were more effective against swimming than swarming. The controls showed that the normal ability of APEC was to remain motile and to form a diameter of 30 mm at 10h and of 40 mm at 24h in swimming tests. Conversely, controls in the swarming zone were recorded as 28 mm at 10h and 35 mm at 24h.

In general, all CT extracts showed a significant impact ($P \leq 0.05$) on swimming. In terms of swarming activity, the CTs of black locust were the only extract that had a significant effect ($P \leq 0.05$) compared to control. It is known that CTs can bind to proteins (Ropiak *et al.* 2017); therefore, it could be possible that CT impact on motility by binding to proteins in flagella structure (O'May and Tufenkji 2011). Moreover, *E. coli* use their flagella to move, hence, if one of these flagella has a problem, the bacterium will stop swimming then fall (Mears *et al.* 2014). On the other hand, during swimming activity, the bacterial cells move relatively independently, but swarming activity requires that bacteria work together which involves bacteria sensing the extracellular signals produced by other bacteria (Sheng *et al.* 2016). Further, these findings supported by the suggestion mentioned by O'May *et al.* (2012) about the relationship between motility and biofilm.

Materials and methods

Plant materials

Three plant materials (yew leaves, tilia flower and black locust leaves) were collected from trees around Reading University/ UK, and dried by air drying at the chemical lab; then the samples were grounded in an impeller SM1 cutting mill (Retsch, Haan, Germany) to pass a <1 mm screen, and stored at room temperature in plastic containers.

Tannin extraction and purification

The samples were extracted and purified by column chromatography on Sephadex LH-20 following the methods of Brown *et al.* (2017). The extractions were, then, frozen, lyophilised, and stored at –20 C° for *in vitro* experiments. Afterwards, these extracts were analysed for CT concentration and composition by thiolysis method with benzyl-mercaptan reaction, which provides the information of CT content (g/100 g extract) and CT composition (mean degree of polymerisation, mDP; procyanidins, PC; prodelphinidins, PD). The PC and PD results are reported on a molar percentage, i.e. % PD + % PC = 100 %) (Gea *et al.* 2011). This reaction was, then, quantified by high-performance liquid chromatography/mass spectrometry (HPLC/MS) to provide further information on mDP and PC/PD and *trans*-flavan-3-ol ratios (Karonen *et al.* 2007).

Bacteriology

The bacterial strain used in these studies was an Avian Pathogenic *Escherichia coli* (strain APEC) belonging to serotype O78 that was isolated from diseased chickens (Alkandhari 2018). This bacterium was stored in Luria-Bertani broth (LB) supplemented with 125 g/l glycerol and maintained at - 80 °C.

Growth and inhibition assays

The growth curve of APEC was determined according to Sheng *et al.* (2016). Overnight APEC cultures were diluted in LB medium to give 1×10^7 CFU/ml, and 200 µl of this mixture which was added to 96 well microtiter plates that supplemented with a range of CT concentrations. The plates were, then, incubated aerobically at 37 °C overnight with shaking at 100 rpm. One row of wells was used per treatment, 6 inner wells of each column were inoculated with bacteria, while the two outside wells of each column were loaded with the positive and negative controls that weather were LB plus the bacterial inoculum without CT, and LB with CT but without the bacterial inoculum. Optimum density values were read hourly at 600 nm using a FluoStar spectrometer (Molecular Device, BMG, Offenburg, Germany). The experiments were repeated three times plus three replicates with fresh culture.

Biofilm formation and cell adhesion of APEC

The effect of CTs on biofilm formation was done as described previously (Shao *et al.* 2015). The same 96 well plates as described above were incubated for 5 days at 25 °C without shaking after the readings had been taken for the growth curve data. After the 5th day of incubation, the content of each well was gently removed, and the wells were washed twice with 150 µl of phosphate buffered saline (PBS) to remove planktonic bacteria. These plates were dried at room temperature for 15 minutes, and adherent bacteria were stained with 150 µl of 1 g/l crystal violet (w/v) for 15 minutes. The wells were, then, rinsed twice with distilled water to remove any residues. After the plates were dried at room temperature, stained adherent cells were detached from the plates using 150 µl of 9:1 ethanol/acetone for 10 min. Then, the optical density (OD) of stained adherent bacteria was determined with the FluoStar spectrometer. The OD was read at 600 nm and the mean OD value obtained from the medium control wells was subtracted from the sample OD values. The formation of biofilm was determined according to the final biofilm formation formulae:

Total OD600 observed – positive control positive with CTs = Final biofilm formation
Three independent experiments were performed in triplicate.

Motility tests for APEC

This assay was performed with different CT concentrations that were tested *in vitro* against APEC using the method previously described (O'May *et al.* 2012). Briefly, swarming and swimming methods were undertaken in Petri dishes containing swarm agar as mentioned by (Kearns 2010). Further, the swim agar supplemented with the same nutrient broth above plus 3 g/l agar poured into Greiner CELLATAR® multi-well culture plates (6 wells plates) as described by (Zhu *et al.* 2015). These plates were left to dry at room temperature, and they were then inoculated with 5 µl aliquots of broth culture that contained different CT concentrations plus bacterial suspension as also the treated groups or broth culture without CTs as control. The inoculum was placed on the centre of the agar surface to enable the visualisation of bacterial motility across the agar surface. Afterwards, these plates were inoculated and taken for growth phase measurements at 37 °C for 10h and 24h. The diameters of the motility zones were recorded.

Statistical analyses

Data obtained from the analysis were processed with Minitab (version 18.0; Minitab software, Inc., PA, USA), which was used to analyse the data via Student's t-tests, ANOVA (one way) and Tukey adjusted comparisons.

The significant differences (*P-values*; the statistical significance was set at P≤0.05) between the control and treated groups were compared. This generated the values for each CT treatment that had an influence on the microbes in growth curve tests and on APEC biofilm formations and motility by ANOVA analysis. All values were based on three replicates (n=3) including control values plus standard error of the means (±SEM).

Acknowledgments

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Conflict of Interest

The authors have no conflict of interest to declare.

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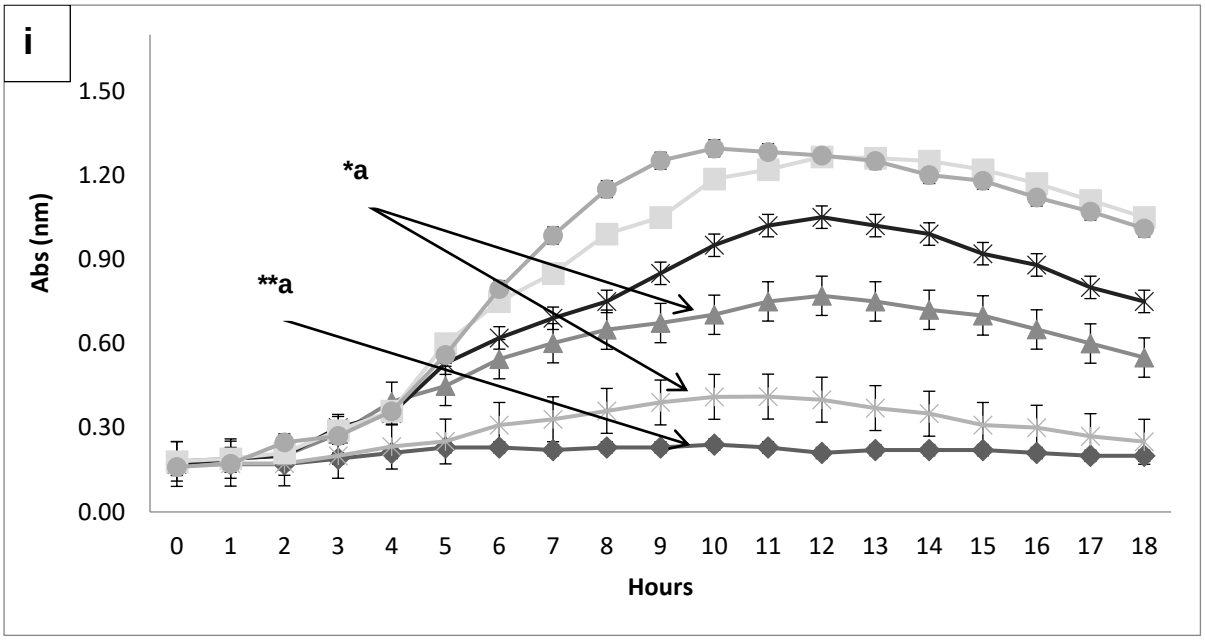
Table 1: The concentration and compositions of CT extracts including mean degree of polymerisation (mDP), prodelphinidins (PD), and *trans*-flavan-3-ols. This table is ordered according to mDP values.

Common name	mDP	PD %*	*trans* %*	CT %**
Yew leaves	7.5 ±0.23	48.4 ±0.55	30.0 ±1.00	93 ±0.75
Tilia flowers	8.9 ±0.35	3.9 ±0.75	2.3 ±1.05	94 ±0.95
Black locust leaves	9.8 ±0.50	76.9 ±0.55	60.3 ±1.00	95 ±0.80

(n=3) ± SEM; %* indicates the molar percentage; %** indicates 1 g CT /100 g extracts.



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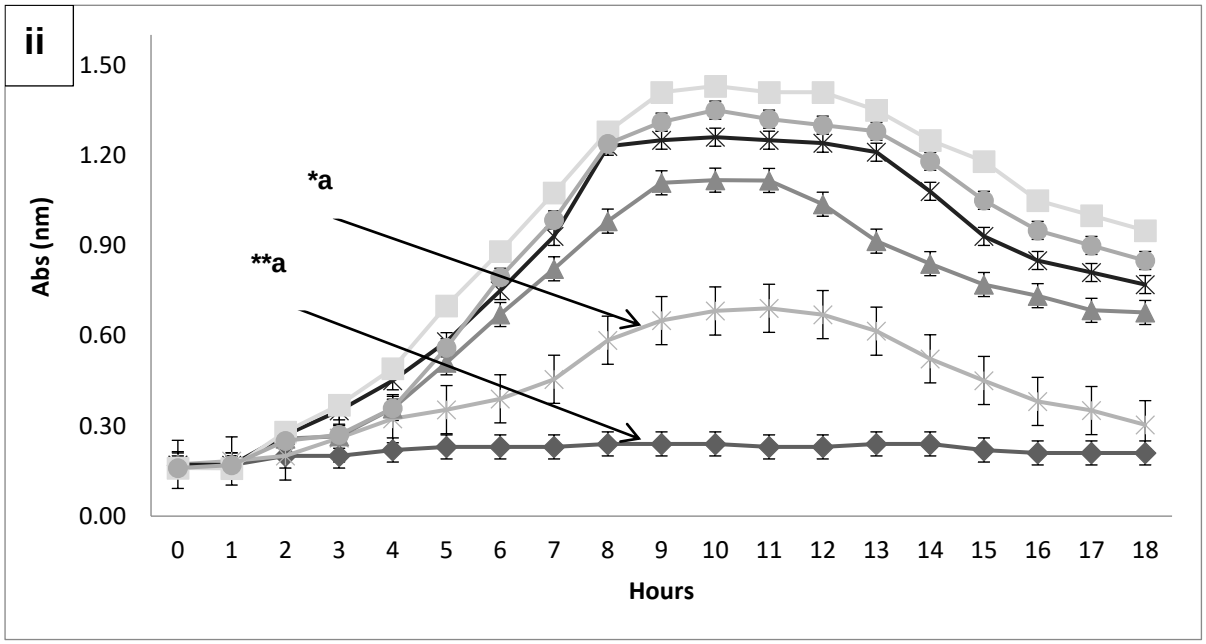

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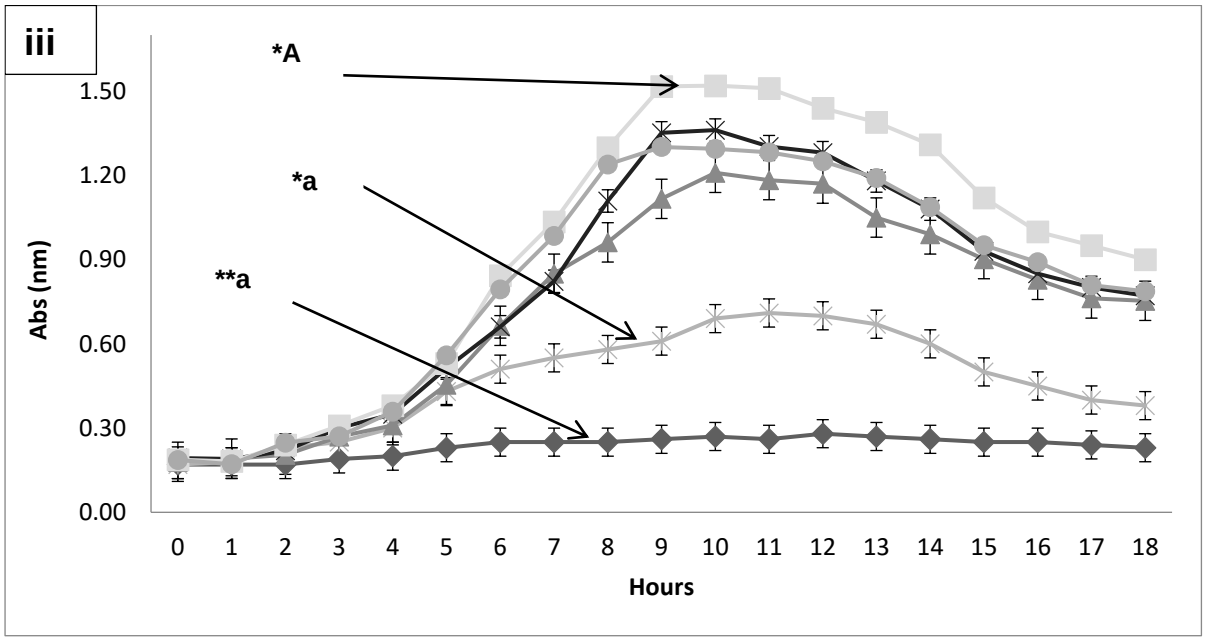

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389 Figure 1: Effect of different concentrations of CTs on growth curves of APEC, including **(i**) black locust
390 (PD-rich), **(ii)** yew (medium levels of PCs and PDs), **(iii)** tilia flowers (PC-rich). (a) indicates decreased
391 growth curve; (A) indicates increased growth curve compared to control; (*) indicates P≤0.05; (**)
392 indicates P≤0.01; n=3 ± SEM. The concentrations of CTs were shown in the figures 10 mg/ml (◊/ black);
393 5 mg/ml (x/ light grey); 2.5 mg/ml (Δ/ grey); 1.25 mg/ml (x/ black); 0.6 mg/ml (□/ light grey); control
394 (○/grey).

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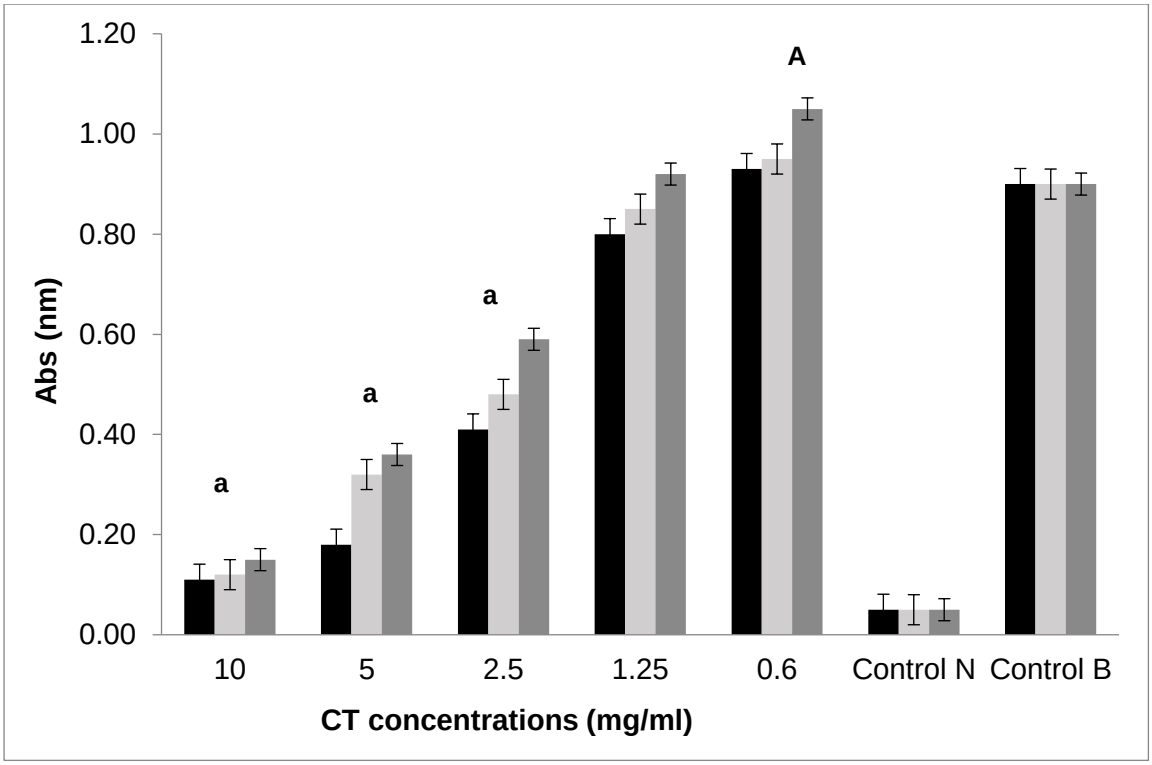

Figure 2: Effect of different CT concentrations on APEC biofilm formation: CTs consisted of prodelphinidins from black locust (black); a mixture of procyanidin/prodelphinidin from yew (light grey); procyanidins from tilia flowers (grey); control N= negative control (LB medium); control B= positive control (bacterial suspension). Significant differences at *P≤0.05*; capital letters indicate an increase and small letters indicate a decrease compared to the positive control (B).

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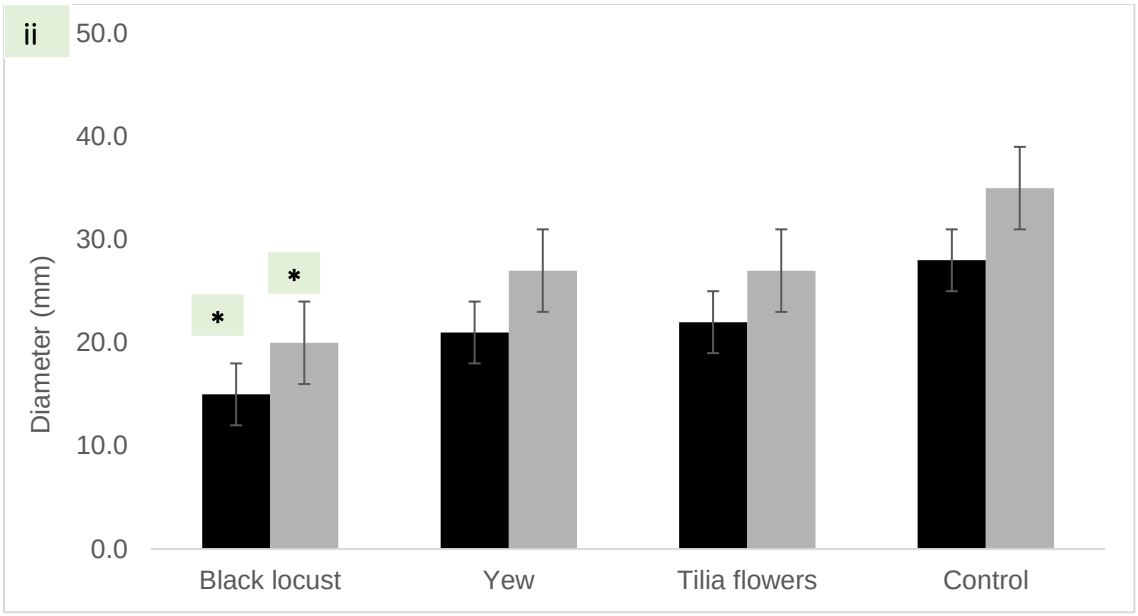

405 Figure 3: Effect of prodelphinidins from black locust, a prodelphinidin/procyanidin mixture from yew and
406 procyanidins from tilia flowers on APEC motility at 10 h (black) and 24 h (grey), including **(i)** swimming
407 activity, **(ii)** swarming activity. (n=3 ± SEM); **(*)** = significant differences at *P≤0.05.*